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CLINICAL INVESTIGATION

Glomerular morphometry in childhood reflux nephropathy, emphasizing the capillary changes

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Glomerular morphometry in childhood reflux nephropathy, emphasizing the capillary changes. As a consequence of nephron loss, reflux nephropathy (RN) causes considerable glomerular hypertrophy. To examine the relative contributions of capillary dilatation and growth in producing hypertrophy, glomeruli contained in unscarred areas of renal biopsies from 19 children with RN were compared with those in 16 children with minimal change nephrotic syndrome and 16 with recurrent hematuria, who were used as controls representing normal childhood growth. Using computerized digitometry we measured the mean glomerular tuft area (GTA) in all complete, undistorted, nonsclerotic glomeruli in periodic acid-Schiff (PAS) stains. Measurements were repeated in four glomeruli of uniform size in periodic acid-silver methenamine stains, the results (GTA_4) correlating significantly with GTA. In the same four glomeruli we measured the mean individual capillary luminal area (CLA) and counted the mean number of lumens per glomerulus (N). Mean mesangial area (MA) was calculated as $GTA_4 - (CLA \times N)$. Cells per distal mesangial region were counted in PAS stains. GTA, GTA_4, N, MA and mesangial cell counts were significantly greater in RN than controls, but CLA and fractional MA (MA/GTA%) did not differ. N correlated highly significantly with GTA_4 in both RN and controls, but CLA did not do so. These findings are consistent with capillary growth by subdivision being the main mechanism of glomerular hypertrophy when nephron loss occurs during childhood, and the identity of the regressions of N versus GTA_4 in RN and controls suggests that compensatory hypertrophy resembles the normal glomerular growth pattern in this age group.

The progession of reflux nephropathy (RN) to chronic renal failure is preceded and accompanied by increasing proteinuria [1, 2]; indeed the severity of proteinuria is one of the best predictors of end-stage renal failure [3]. Kincaid-Smith first drew attention to the presence of focal and segmental glomerular sclerosis in adult patients with RN who were proteinuric [2, 4], demonstrating that such lesions occurred in radiologically unaffected contralateral kidneys. In a previous study of renal biopsies obtained from 24 children and adolescents with RN, we confirmed her findings and demonstrated a highly significant correlation between the severity of segmental glomerulosclerosis and the amount of proteinuria [5]. In the same paper we also emphasized that the sclerotic lesions invariably originated at the hilum, as had been observed in Wistar rats following subtotal nephrectomy [6], and we reported previously undescribed hilar vascular changes.

In a morphometric study of the same 24 biopsies, using as controls 13 children with benign recurrent hematuria (RH) and 6 with minimal change nephrotic syndrome (MCNS), all with normal glomeruli, we demonstrated that the nonsclerotic glomeruli in RN showed a mean tuft area approximately double that of controls [7]. There were significant inverse correlations between glomerular size and both residual radiological renal parenchyma area and glomerular filtration rate (GFR). By comparing our results with those of El Khatib, Becker and Kincaid-Smith [8] in adults with more advanced disease, we concluded that the compensatory glomerular hypertrophic response was more vigorous in children. This accords with the finding that compensatory renal hypertrophy and functional enhancement following nephrectomy are greater in young rats than in mature ones [9]. Thus the extensive renal scarring that RN can cause may be perceived as a human counterpart of experimental renal ablation in the rat.

It has been consistently demonstrated in animal experiments [10–12], as well as in humans with loss of functioning renal mass [7, 8, 13], that glomerular hypertrophy both precedes and accompanies the development of sclerosis. In our previous study of RN there was a highly significant correlation between glomerular size and the extent of segmental glomerulosclerosis [7]. Morphometric analyses of the glomerular capillary alterations associated with compensatory hypertrophy in rats reveal evidence of dependence upon the age at which nephrectomy was undertaken, the extent of renal ablation and the presence or absence of arterial hypertension [14–18]. The capillary dilatation which is a feature of 5/6 nephrectomy in adult rats [14–16] appears to be lacking in infant rats subjected to mononephrectomy, in whom increased total length [17, 18] and an increase in number [18] have been reported. We are unaware of any comparable data in human material, and the present investigation was designed to examine the comparative roles of capillary dilatation and growth in producing glomerular enlargement.

Methods

Reflux nephropathy

Of the 24 patients investigated previously [5, 7], in the present study we included 19 who were less than 16-years-old, since we did not have access to control material above this age. The age at biopsy ranged from 5.2 to 15.5 (mean 11.9 ± 2.9) years (Table 1). Thirteen were girls. Vesicoureteric reflux was bilateral in 11 and unilateral in 4; the remaining 4 children had solitary kidneys with severe reflux. Two children were hypertensive, but in one the blood pressure was well controlled with treatment at the time of

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Table 1. Clinical and glomerular morphometric findings in 19 children with reflux nephropathy and 32 controls

	Reflux nephropathy	Controls	*P*
Sex (M:F)	6:13	22:10	<0.05
Age at biopsy *years*	11.9 ± 2.9	8.1 ± 3.7	< 0.001
Body surface area m^2	1.30 ± 0.28	1.0 ± 0.30	< 0.001
Glomerular morphometry μm^2			
Tuft area			
PAS, all glomeruli (GTA)	17797 ± 10386	8341 ± 1932	< 0.001
GTA/m^2 BSA	14316 ± 8463	8739 ± 3212	<0.02
PASM, 4 glomeruli (GTA_4)	21794 ± 9236	10848 ± 2430	< 0.001
Capillary luminal area (CLA)	95.8 ± 22.5	84.4 ± 13.4	NS
No of lumens per glomerulus (N)	106.0 ± 38.0	58.6 ± 12.6	< 0.001
Mesangial area (MA)	11490 ± 5974	5744 ± 1528	< 0.001
Fractional MA (MA/GTA%)	51.9 ± 8.1	52.6 ± 5.4	NS
Cells/distal mesangial zone	1.56 ± 0.22	1.40 ± 0.12	<0.01

Results are given as mean ± SD.

biopsy. In 15 patients the GFR, measured by ^{51}Cr EDTA clearance, was >60 ml/min/1.73 m^2, and in the remaining 4 it ranged from 50 to 60 ml/min/1.73 m^2. Eight patients had proteinuria, defined as a protein/creatinine ratio (U_P/U_{Cr}) >20 mg/mmol [19], and in three instances proteinuria was in the nephrotic range (U_P/U_{Cr} >200 mg/mmol).

Controls

These consisted of biopsies obtained from 16 children with RH and 16 with MCNS, their ages at biopsy ranging from 1.8 to 15.2 (mean 8.1 ± 3.7) years (Table 1). Only 10 were female, because of the male predominance in RH and MCNS; therefore the sex distribution was significantly different from that of patients (χ^2 = 5.24, $P < 0.05$). Their morphometric measurements are reported in detail elsewhere, and are considered to represent normal glomerular growth during childhood [20]. Seven MCNS controls had proteinuria at the time of biopsy, which in five instances was in the nephrotic range as previously defined [21]. Eleven children with RH presented with recurrent gross hematuria and five with microscopic hematuria, although at the time of biopsy 11 had normal urine. All controls were normotensive and had normal renal function.

Renal biopsies

The biopsies were obtained from RN and control patients by standard technique, using a Tru-Cut needle to minimize glomerular artifacts [22], during the period 1985 to 1991. After removal of tissue for immunofluorescence (IF) and electron microscopy (EM), specimens were immediately fixed in alcoholic Bouin's solution for five to six hours, followed by overnight fixation in 10% buffered formalin, and were embedded in paraffin wax. Sections were cut at 2 μm and stained with haematoxylin and eosin (HE), periodic acid-Schiff (PAS), and Lendrum's martius scarlet blue, and at 1 μm and stained with periodic acid-silver methenamine (PASM). All control glomeruli were optically normal, apart from rare obsolescent glomeruli consistent with MCNS [23]. IF was essentially negative and EM was normal apart from variable epithelial foot process fusion in a few MCNS patients biopsied during relapse.

Fig. 1. *Representative tracing of a whole glomerular tuft (top) and the individual capillary lumens of the same tuft.* (Modified from Fig. 1 of reference 20; used with permission of John Wiley and Sons, Ltd.).

Glomerular morphometry

The basic techniques have been described in detail previously [7, 20]. Glomeruli that were either incomplete, distorted, globally sclerosed, or tangentially sectioned were excluded from analysis. Sclerotic and nonsclerotic glomeruli which were found in rare bands of fibrous scar tissue in six of the RN biopsies were also excluded. Microscopic images of all the remaining glomeruli in 2 μm PAS stains were projected using a ×25 objective lens at a final magnification of ×240, and measured by tracing outlines of the glomerular tufts on the digitizer tablet of a Kontron: MOP

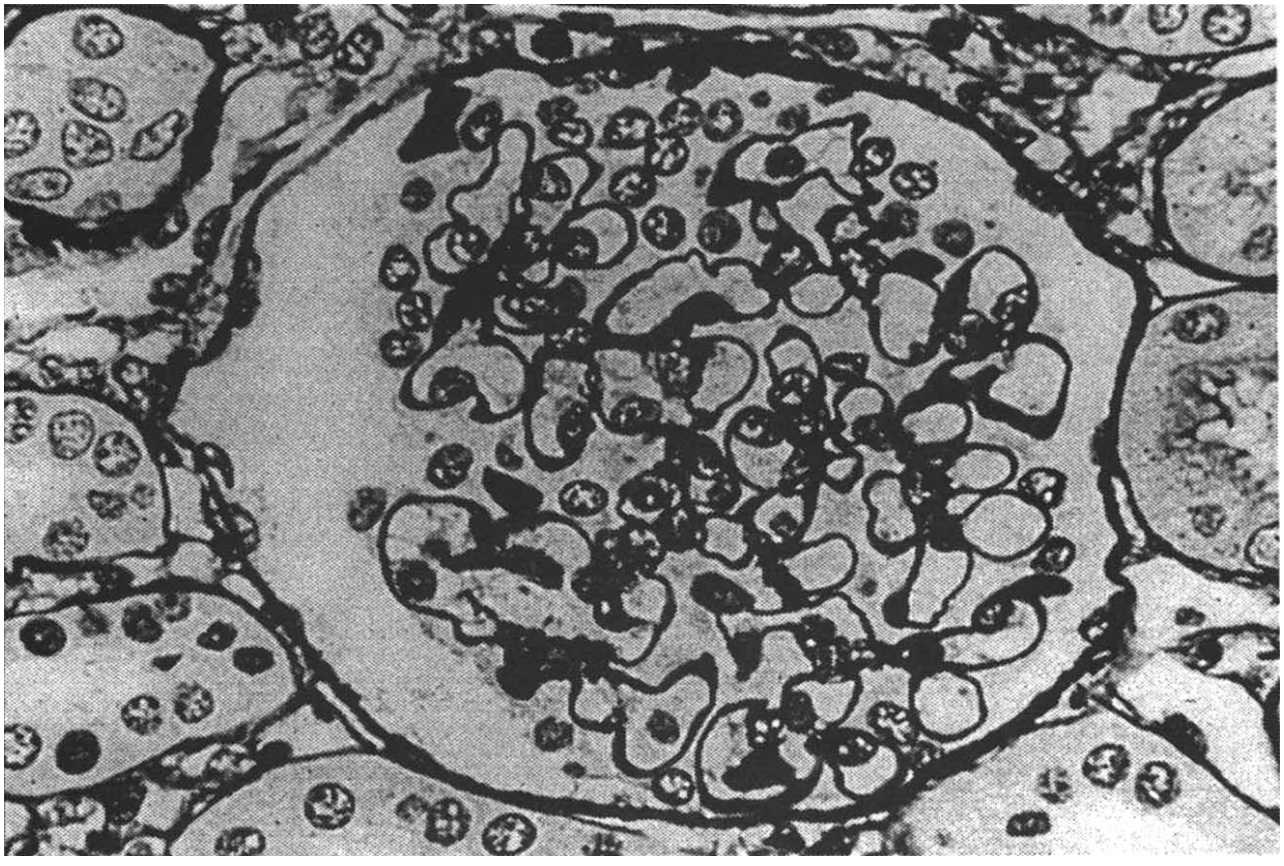

Fig. 2. *The glomerulus which provided the tracings in Fig. 1, stained with periodic acid-silver methenamine.* The capillary loops, mesangial regions and podocytes are clearly defined.

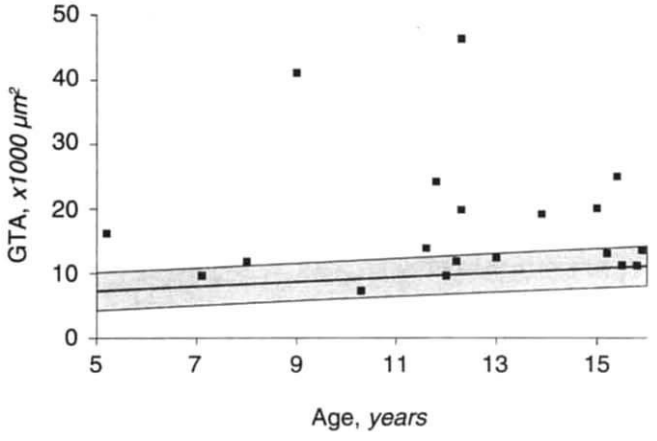

Fig. 3. *Relationship of mean glomerular tuft area (GTA) to age at biopsy in RN.* In contrast to the 95% confidence limits for the 32 controls (shaded area), there is no correlation.

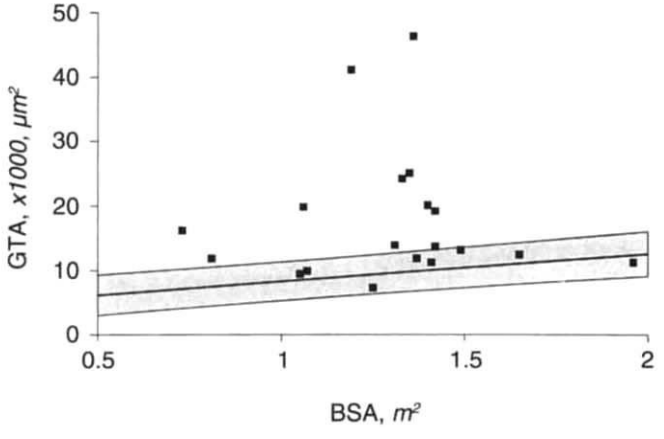

Fig. 4. *Relationship of mean GTA to body surface area (BSA) in RN, showing a lack of correlation, in contrast to the 95% confidence limits for controls.*

videoplan computerized image analyzer. Podocyte nuclei and any visible cytoplasm were excluded from the tracings. Simultaneously projected 200 μm linear scales were also drawn, and the computer was programmed to measure the area contained within each complete outline. From these data the mean glomerular tuft area (GTA) of each biopsy was calculated.

Employing the same technique, but using 1 μm PASM stains to facilitate delineation of basement membranes, and a $\times$40 objective lens with a final magnification of $\times$390, all the capillary lumina contained in four glomeruli per biopsy were traced (Fig. 1). These were selected so that the glomerular tuft diameter did not vary by more than one-third, thereby avoiding tangential

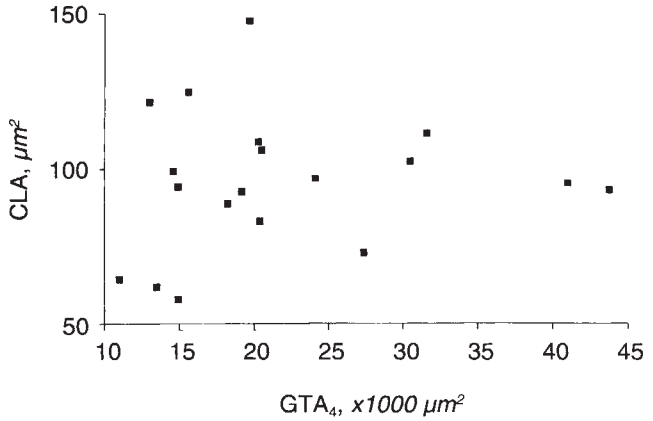

Fig. 5. *Relationship of mean capillary luminal area (CLA) to GTA_4 in RN.* There is no correlation, as also observed in controls.

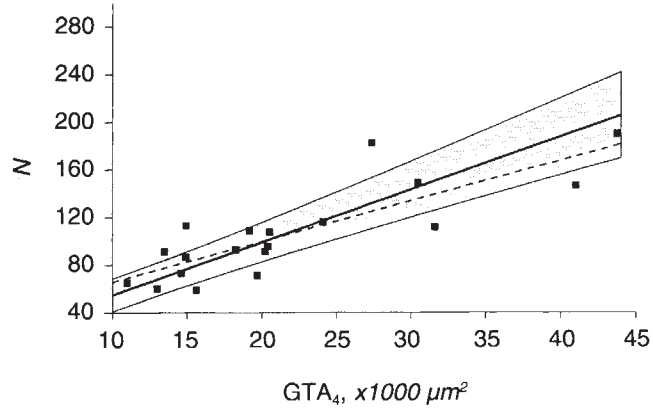

Fig. 6. *Correlation of the mean number of capillary lumens per glomerulus (N) with GTA_4 in RN, compared with the 95% confidence limits for controls.*

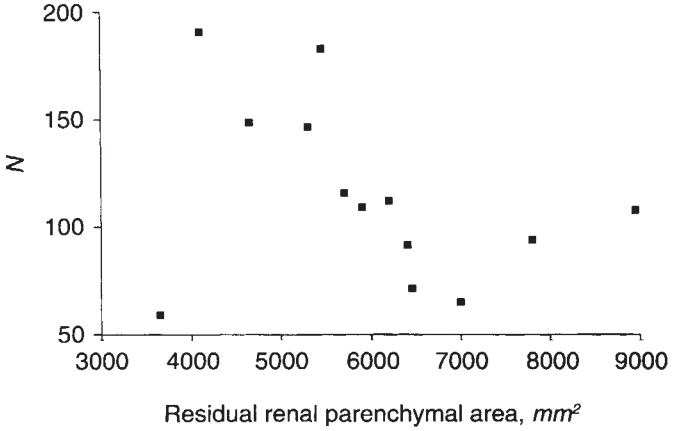

Fig. 7. *Relationship of the mean number of capillary lumens per glomerulus (N) in RN to residual renal parenchymal area, obtained from intravenous urogram tracings [20].* The trend towards a negative regression failed to reach significance.

Table 2. The interrelations between the glomerular morphometric determinations, and with body surface area, in reflux nephropathy and controls (*P* values)

	Reflux nephropathy				Controls			
	GTA_4	CLA	N	MA	GTA_4	CLA	N	MA
BSA	NS	NS	< 0.001	< 0.001	< 0.001	NS	< 0.001	< 0.01
GTA_4		NS	< 0.001	< 0.001		NS	< 0.001	< 0.001
CLA			NS	NS			NS	NS
N				< 0.001				< 0.001

NS is not significant.

sections [24], except in one RN biopsy specimen which contained only six very large glomeruli. At least one of the four glomeruli was selected from each of the two cores of tissue obtained at biopsy, and none showed either segmental or global sclerosis. From these tracings the mean individual capillary luminal area (CLA) was calculated, while the number of capillary loops traced, ranging from 162 to 337 per biopsy was recorded by the computer and the results expressed as the number per glomerulus (N). In order to calculate mesangial area, the whole tufts of the same four glomeruli were also traced, to yield the glomerular tuft area (GTA_4). There was a highly significant correlation between GTA_4 and GTA in the 32 controls ($r = 0.812$, $P < 0.001$), thus validating the method [20]. Since the podocytes were excluded from all tracings, while the sparse endothelial nuclei were included in the capillary loop tracings (Fig. 2), it was possible to estimate the total mesangial area (MA) for each glomerulus as the difference between the whole tuft area and the sum of the capillary luminal area [20]: $MA = GTA_4 - (CLA \times N)$. The fractional mesangial area is expressed as $MA/GTA_4\%$.

Mesangial cellularity

Mesangial cellularity was assessed semiquantitatively by counting the number of cell nuclei present in each individual distal mesangial zone in PAS sections at a magnification of ×800, using a 100-point eyepiece graticule to avoid duplicate counting. A minimum of 100 mesangial regions were counted in at least 10 glomeruli, avoiding those immediately adjacent to the hilum, where cellularity is normally greater. The results are expressed as cells per distal mesangial zone. This method does not yield the absolute and fractional mesangial cell volumes per glomerulus [17], but nevertheless affords a comparison of relative cellularity between RN patients and controls, in sections of uniform thickness [25].

Statistical analysis

Tabulated data are presented as means ± SD. Differences between RN patients and controls were analyzed using either Student's or Welch's *t*-test, according to the dispersion of data. Correlation coefficients were calculated by Pearson's method and differences between the regression lines of the two groups were tested by analysis of covariance. Differences were considered significant for $P < 0.05$. In the Figures the data from the 32 controls are presented as the 95% confidence limits of the regressions.

Results

The RN patients, as a group, were significantly older than controls (Table 1), although there was appreciable overlap in the 5 to 15 year age range. The mean body surface area (BSA) of RN patients was correspondingly greater. The glomerular yield per

biopsy ranged from 13 to 130 (mean 51) for controls and 6 to 92 (mean 31) in RN. Eight RN biopsies showed segmental sclerosis affecting from 1 to 39% of glomeruli, and 10 had global sclerosis in 1 to 33% of glomeruli. There was no significant difference in GTA between glomeruli with and without segmental sclerosis.

The mean GTA in RN was significantly greater than that of controls. Even when allowance was made for the younger age range of controls by correcting GTA for BSA, the glomeruli in RN were 64% larger than in controls. Likewise GTA_4 in RN was significantly greater than in controls.

Figure 3 shows the relationship between GTA and age at the time of biopsy. Data from the 32 controls are shown as the regression $y = 361.7x + 5406.0$ ($r = 0.698$, $P < 0.001$), together with 95% confidence limits. In RN patients there was no correlation between GTA and age ($r = -0.019$, NS). It can be seen that more than half the RN plots lie above the upper limit of controls. Similarly, Figure 4 displays the relationship between GTA and BSA, and again there was no correlation in RN patients, whereas the controls showed a highly significant regression: $y = 4291.3x + 4032.7$ ($r = 0.672$, $P < 0.001$).

Although the mean CLA in RN was slightly greater than in controls (Table 1), the difference was not statistically significant. Moreover, there was no correlation between CLA and GTA_4, in either controls or RN (Fig. 5). In contrast, the mean number of capillary lumina per glomerulus (N) in RN was almost double that of controls (Table 1), and there was a highly significant correlation with GTA_4 (Fig. 6) in both controls ($y = 0.0044x + 10.3$; $r = 0.855$, $P < 0.001$) and RN ($y = 0.003x + 31.7$; $r = 0.828$, $P < 0.001$). Although Figure 6 shows that the dispersion of N about the mean was wider in RN than in controls, analysis of covariance revealed no significant difference between these regressions.

The relationship between the number of capillary loops per glomerulus and the loss of functioning renal tissue in patients with RN was explored using the radiological data previously obtained from them by Yoshiara et al [7]. The residual renal parenchyma area was measured digitometrically by subtracting the area of the pelvicalyceal system from that of the complete renal outline in tracings made from intravenous urogram films obtained immediately prior to renal biopsy. Adequate tracings were available for 13 of the 19 patients in the present study. Although there is a tendency for N to increase with diminishing residual renal parenychmal area (Fig. 7), the regression failed to reach significance ($r = -0.352$, $P > 0.1$).

The mean calculated MA was significantly increased in RN, compared with controls; however, fractional MA (MA/GTA%) was not significantly different (Table 1). The mean number of cells per distal mesangial zone was significantly greater in RN than controls (Table 1). In controls no individual distal MA contained more than three nuclei, but 12 RN biopsies showed mild focal mesangial hypercellularity, with counts of up to six nuclei per distal mesangial region.

The complex interrelations between these glomerular morphometric determinations, and with BSA, in RN and controls, are summarized in Table 2.

Discussion

Digitometry is an exacting and time consuming procedure [26], and in the present study involved the tracing of an average of 250 capillary lumina per biopsy in order to measure CLA. Our decision to measure only four glomeruli was justified by the narrow dispersion of morphometric data obtained from each individual glomerulus, the minimal interglomerular differences and the highly significant correlation of GTA_4 with GTA in the 32 controls [20], indicating that the technique was representative. The methodology used may not reflect true glomerular size, which necessitates the identification of maximum glomerular diameter in serial sections in order to calculate glomerular volume [12, 27], but the limited amount of human biopsy material available following routine diagnostic procedures made this impractible. Whereas previously we used age-matched controls [7], in the present study we were able to use the comparatively narrow 95% confidence limits derived from a larger number of controls, spanning a wider age range, which we consider to represent the normal glomerular growth pattern during childhood [20]. By adjusting GTA for BSA we were able to make valid comparisons, and thereby confirmed earlier work [7] demonstrating that the glomeruli in RN are considerably larger than normal for age.

We extended our morphometric observations in the present study by additionally investigating the capillary and mesangial changes. Again, we were not concerned with true capillary diameter, since a proportion of capillary loops seen in section are cut obliquely. Our aim was to observe the differences in mean size and number of lumens between RN patients and controls. Our results clearly demonstrate that the number per glomerulus in RN was nearly double that in controls, and proportional to the increase in tuft size, whereas the luminal area was only marginally greater, and not significantly so. These results are comparable with the experimental findings of Olivetti et al [17] and Nyengaard [18], who demonstrated relatively minor increases in capillary radii but large increases in total capillary length, compared with controls, when mononephrectomy was undertaken in infant rats. In contrast, Bidani et al [14] and Daniels and Hostetter [15] found appreciable capillary dilatation following 5/6 nephrectomy in normotensive and hypertensive adult rats, respectively. It is therefore possible that the mechanisms of compensatory glomerular hypertrophy differ in growing and mature animals. All but two of the RN patients in the present study showed radiological renal scarring when first investigated. In a prospective study of children with vesicoureteric reflux [28], there was a 57% prevalence of existing scarring on initial investigation which was equalled in the infant subgroup, whereas new scarring did not develop after the age of seven years. Thus RN is essentially a disease of early onset, and a different model would be required to study the glomerular capillary changes of remnant kidney in the human adult.

We previously demonstrated a highly significant negative correlation between glomerular tuft size and residual renal parenchymal area [7], and the present study reveals a similar trend with regard to capillary numbers per glomerulus, as would be expected, although this failed to reach statistical significance, presumably owing to the small numbers and wide dispersion of data. The absolute expansion of mesangial area observed in RN was considerable, but the fractional mesangial area did not differ from controls, indicating that this increase is merely proportional to overall glomerular hypertrophy, whether compensatory or due to normal growth. This accords with the results of quantitative morphometric analysis of the mesangium reported in both normotensive and hypertensive adult rats in response to 5/6 nephrectomy [16], and with the observations of Nagata, Schärer and Kriz [29] following uninephrectomy in young rats, but differs from the

results obtained in young rats by Olivetti et al [17], who observed a disproportionate mesangial matrix response. However, the limited biopsy material available necessitated the employment of methodology whose accuracy could not match that of point counting in multiple ultramicroscopic preparations, which permits the measurement of absolute volume [16, 17]. Our findings throw no further light on why the segmental sclerosis associated with remnant kidney always originates at the hilum, as has been consistently demonstrated in both animal experiments [6, 30] and humans with reduced renal mass [31–33]. A likely explanation, proposed by Daniels and Hostetter [15], is that the greater diameter of glomerular capillaries at the hilum [34] implies correspondingly greater tension, in accordance with Laplace's law, and this may carry an increased risk of capillary wall damage with consequent leakage and accumulation of particulate matter into the hilar mesangium, as has been demonstrated experimentally [30]. The recent observation by Nagata et al [29] that dilated and abnormally-shaped capillaries developed in the vicinity of the vascular pole following uninephrectomy in young rats also has relevance to this hypothesis, as does our own previous finding of numerous subendothelial hyaline deposits in hilar arterioles of increased luminal diameter [7, 20].

The highly significant correlation between the number of capillary lumens per glomerulus and glomerular size, observed in both RN and controls (Fig. 6), is of particular interest. The statistical identity of the regressions for patients and controls suggests that the compensatory hypertrophy observed in RN of juvenile onset closely resembles the normal pattern of childhood glomerular growth. In this respect it is of interest that transgenic mice, which express excessive growth hormone levels, are prone to age-related premature glomerular hypertrophy and sclerosis [11]. Conversely, El Nahas et al [35] demonstrated in growth hormone-deficient dwarf rats that, following unilateral nephrectomy, hypertension, proteinuria and raised plasma creatinine levels developed more slowly than in adult male Wistar rats, while at sacrifice 120 days postoperatively the glomeruli showed less hypertrophy and sclerosis. It is also relevant that Rosendahl et al [36] demonstrated increased serum and kidney tissue levels of insulin-like growth factor-II (IGF-II) in infant rats following unilateral nephrectomy, whereas normally serum levels decline early in postnatal life as they are gradually replaced by IGF-I. Bhathena et al [13] observed that two patients with congenital solitary kidneys and one with oligomeganephronia developed larger glomeruli with more sclerosis than one who acquired unilateral disease in adulthood. Moreover, our previous study [7] indicated that the mean glomerular capsular area in 24 children with RN whose mean age was 12.9 years was greater than that of the adult patients investigated by El Khatib et al [8]. Whereas compensatory renal growth in adult rats is mainly related to cellular hypertrophy, in infant rats hyperplasia plays a major part [37, 38]. This is in keeping with the mesangial hypercellularity observed in RN in the present study, even though it was not of sufficient degree to meet the criteria of diffuse proliferation [25].

The increased number of capillary lumens observed in section in association with glomerular hypertrophy, whether due to normal childhood development or in compensation for nephron loss, could technically be due to either elongation and increased tortuosity or subdivision by branching. Remuzzi et al [39] calculated, from three-dimensional reconstruction of the glomerular capillary network in Munich-Wistar rats, that 11.8% of the capillary surface is at filtration equilibrium at any time. While it cannot be assumed that this necessarily applies to the human kidney, there is at least a theoretical suggestion that elongation of capillaries would confer no advantage, whereas branching would yield a greater number of short capillaries, which, together with an increased blood flow, would favor hyperfiltration. We were unable to investigate these two possibilities further with the limited biopsy material available. However, our results are in keeping with those of Nyengaard [18], who performed unilateral nephrectomy during the neonatal period in female Wistar rats, and found approximately double the number of capillary lumens per glomerulus, with only 8% mean increase in luminal diameter. By tracing capillaries through serial sections and applying Euler numbers he was able to conclude that capillary subdivision had occurred, which, in the light of our results, suggests that this is a possible mechanism of compensatory glomerular hypertrophy when severe nephron loss is sustained during childhood.

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